

(51) International Patent Classification 7 : A61K 7/06, 7/48, 7/46, 7/13, 47/48		A1	(11) International Publication Number: WO 00/48558
			(43) International Publication Date: 24 August 2000 (24.08.00)
(21) International Application Number: PCT/GB00/00517 (22) International Filing Date: 17 February 2000 (17.02.00) (30) Priority Data: 9903584.2 18 February 1999 (18.02.99) GB (71) Applicant (for all designated States except US): UNIVERSITY OF LEEDS [GB/GB]; Leeds LS2 9JT (GB). (72) Inventor; and (75) Inventor/Applicant (for US only): FINDLAY, John [GB/GB]; Dept. of Biochemistry & Molecular Biology, The University of Leeds, Leeds LS2 9JT (GB). (74) Agent: HARRISON GODDARD FOOTE; Tower House, Merriion Way, Leeds LS2 8PA (GB).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: MODIFIED CALYCINS			
(57) Abstract			
A calycin comprises a binding domain for binding to at least one selected agent and a targeting domain that binds to at least a part of a hair fibre or skin surface and can be used to target said calycin to the hair fibre or skin surface. The invention also provides a hair or skin care composition comprising at least one calycin.			
BEST AVAILABLE COPY			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## **MODIFIED CALYCINS**

The invention herein described relates to the use of calycins, and in particular, the use of lipocalins in the hair and skin care industry, and more particularly to the transport and/or binding of ligands to hair fibres and/or skin and also the modification of lipocalins to alter the specificity of said lipocalins for said hair fibres and skin and/or said ligands.

Lipocalins are a diverse family of extracellular proteins found in biological organisms. They display various functions related to the binding and transport of ligands. For example, they are involved in mediating pheromone activity, olfaction, taste, vision, immunomodulation and general functions relating to cellular homeostasis.

Arguably the most extensively studied lipocalin is the retinol binding protein (RBP) which transports retinol around the body. Retinol (or vitamin A) is an extremely important substance which is crucial to the normal functioning of the visual, the reproductive and the immune systems, and in haematopoiesis. Metabolites of retinol are also active in development, differentiation and against cancer cells.

During embryonic development, a deficiency in vitamin A (retinol) can lead to tetragenic effects on the development of the nervous system, eyes, face, teeth, ears, limbs, skin, lungs and the urinogenital system. The importance of maintaining the optimal concentration of retinol is also shown by the effects of excess retinol on the developing embryo and adult. This too can lead to abnormal development of many organs and to toxic effects leading to disability and death.

The body stores retinol in the liver cells from which it is mobilised when required. RBP complexes with retinol in the cell and the complex is secreted into the plasma from where it can reach almost all tissues. RBP is not freely diffusible in the plasma

but is associated with a further protein, transthyretin (TTR). TTR has several functions including retaining RBP in the plasma. RBP also interacts with a further protein bound at the cell surface of tissues targeted for retinol, the so called RBP receptor. The binding of RBP to this receptor induces the release and uptake by the cell of retinol, which can then be utilised for a large variety of metabolic and regulatory processes. The receptor has now been cloned and encodes a polypeptide of 63kDa ( see US 5,573, 939 and US 5 573 939). RBP is therefore a very versatile protein, being able to interact with retinol and at least two macromolecules in a highly specific manner.

The 3-dimensional structure of RBP was determined in 1984 and revealed a novel structure. As many more lipocalins were identified and characterised, it became apparent that this basic eight- $\beta$ -stranded structure was conserved in all the structural homologues although the overall level of amino acid sequence identity is low. This family of proteins became known as the lipocalins (derived from the Greek word lipos, meaning fat and calyx meaning cup). A closely related family of 10- $\beta$ -stranded intracellular protein has also been found, the two groups together comprising the calycins.

The lipocalins are found throughout biological life and range in molecular weight from approximately 18kDa to 45kDa. However, this represents the monomeric molecular form; many lipocalins exist in as multimers. For example the bilin-binding protein exists as a homotetramer of a 19.5kDa monomeric subunit. Apolipoprotein D exists as a dimer, but is also associated with other proteins. RBP is found complexed with TTR in up to a 1:1 ratio, while crustacyanin appears to contain 16 subunits in its stable molecular form.

As mentioned previously, the lipocalins are not highly conserved at the amino acid level, but do retain certain structural features that make them recognisable as lipocalins. The core structure is represented by orthogonally arranged  $\beta$ -sheets, the

$\beta$ -strands connected to each other to form a barrel-like structure closed at one end, thereby producing the 'cup shaped' structure. The cavity thus created represents the binding pocket for the ligand transported/bound by the lipocalin. There are some short regions of sequence homology that help to identify a protein as a lipocalin. The two most definitive but not obligatory being the GXW and TDY motifs.

The major function of the lipocalins is the binding and transport of specific ligands (although at least one has enzymic activity) which are usually small hydrophobic molecules. The specificity of binding is determined by the conformation and constituent side-chains of the lipocalin pocket. It is of note that *in vitro* many lipocalins can bind with high affinity to a range of hydrophobic molecules not normally encountered in nature. This may represent an inherent ability of the lipocalins to bind molecules having particular biochemical and structural properties.

The realisation that RBP binds not only retinol and TTR, but also the membrane-associated retinoic acid receptor, indicates that RBP contains specific domains that enable it to interact with cellular targets with relatively high affinity and specificity. Indeed, we have found that one can alter the specificity of a lipocalin by genetically engineering into same regions involved in receptor recognition so as to target a lipocalin (and a ligand) to a specific tissue or site in the body.

Another example of a lipocalin which has been extensively studied is  $\beta$ -lactoglobulin.  $\beta$ -lactoglobulin is a very abundant protein found in the milk of mammals. The monomer molecular weight of bovine  $\beta$ -lactoglobulin is 18kDa, corresponding to 162 amino acids. A number of investigators have shown that  $\beta$ -lactoglobulin binds retinol and fatty acids *in vitro*. However, the exact role played by  $\beta$ -lactoglobulin *in vivo* is still not understood.

We investigated the potential of  $\beta$ -lactoglobulin to bind fatty acids at a non-native site. The term non-native is defined as a targeting site not naturally encountered by a

lipocalin. The hair cuticle is thought to be coated in fatty acids, these fatty acids may function as a hydrophobic barrier to water and they also give hair its natural sheen and texture. Cosmetic hair conditioners function to accentuate these features of hair. However, conditioners currently available only have a transient association with the hair cuticle and therefore the user has to periodically apply conditioner to maintain the sheen and body of the hair.

We demonstrate that lipocalins, for example  $\beta$ -lactoglobulin, can be adapted to provide a conditioning property to hair. The majority of hair products contain protein which is claimed to imbue the hair with body and protective elements. However, the ability of these protein additives to bind human hair with high affinity is questionable.

In contrast the affinity of  $\beta$ -lactoglobulin for the fatty acids that coat hair cuticles allows a lipocalin based conditioner to be formulated which binds tightly to hair and imparts a variety of desirable properties. In addition lipocalins that bind hair can impart other beneficial features to hair by altering, for example, the charge on the protein to alter its hydrophobicity thereby conferring body to the hair fibres it binds. Further, lipocalins that bind hair may be post-translationally modified ( eg glycosylation, lipidation) to alter the feel and/or sheen of hair bound by the modified lipocalin. Alternatively, lipocalins can be adapted to carry a variety of agents to hair to provide an indirect conditioning and protective effect.

Additionally or alternatively multimeric complexes of lipocalins can be adapted to carry more than one beneficial agent to hair. For example, and not by way of limitation, multimeric lipocalin complexes can be used to carry both conditioning agents and fragrances to hair. Fragrance molecules are generally volatile. The binding of fragrance molecules to a lipocalin will provide for delayed and/or controlled release. One example of such a lipocalin is the pyrazine-binding protein which binds bell-pepper odourant 3-isobutyl-3-methoxypyrazine. Alternatively lipocalins can be selected from libraries to bind an odourant of choice.

Also, other haircare products can be developed with multi-functional properties. For instance, the fatty acid binding domain of one lipocalin may be complexed with the ligand binding domain of another lipocalin.

In this respect the pigmented lipocalin, crustacyanin, has also been sequenced and modelled by us. The ligand in this instance is a carotenoid, astaxanthin, and there are a number of such lipocalin-carotenoid complexes in nature. The interaction between carotenoid and lipocalin produces a change in the absorbance characteristics of the astaxanthin, such that the complex now assumes a different colour. It is, therefore, proposed that such coloured complexes may be engineered for use as specific hair colorants.

Also in recent years, hair mite has become a common problem in schools, mainly due to the phasing out over the last 15-20 years of head inspections in schools by nursing staff. Currently, treatment of hair mite infections is by administration of an insecticidal shampoo (pyrethroid based) which is effective at removing the primary infection, but does not provide prophylactic treatment (and has given grounds for safety concern). Therefore, re-infection is common, leading to rounds of treatment using expensive (and questionable) insecticidal shampoos. It would therefore be highly desirable to produce shampoo for prophylactic treatment which has long lasting effects based on the tight binding and the slow release of insecticidal agents preferably of low concentration. Such formulation may also have value as insect repellents.

Moreover animals, both domestic and/or livestock, suffer greatly from various parasitic infections of the outer surface of the body. It would therefore be highly desirable, to produce anti-parasitic complexes that remain bound to fibres of fur, hide, or feathers to prevent and/or cure parasitic infections, preferably by the slow release of toxic agents.

It is therefore an object of the invention to provide a calycin or calycin complex for use in the treatment of hair.

It is a further object of the invention to provide a calycin or calycin complex for use in the delivery of agents which prevent and/or cure various hair and/or scalp conditions.

In its broadest aspect the invention concerns the modification of either, or both, the ligand binding domain and/or targeting domain of a calycin in order to provide for the selective delivery of at least one ligand to a hair fibre and/or the skin surface.

According to a first aspect of the invention there is provided a calycin comprising a binding domain for binding at least one selected agent and a targeting domain that binds to at least a part of a hair fibre and/or skin surface for targeting said calycin to said hair fibre and/or skin surface.

Reference herein to the term targeting is intended to include, without limitation, the preferential or selective location or binding of said calycin to said hair fibre and/or skin surface.

In a preferred embodiment of the invention said calycin further comprises a subunit interaction domain that allows for the multimeric assembly of said calycins. In this respect said subunit interaction domain may be a naturally occurring part of said calycin, or alternatively, it may be inserted with same using conventional synthetic or recombinant techniques.

It will be apparent to the skilled artisan that said binding domain and/or said targeting domain may be endogenous to said calycin, however, in the instance where said calycin would not naturally bind a hair or skin treatment product or a hair fibre and/or skin surface, the corresponding binding and/or targeting domain is adapted



accordingly. This adaption may comprise either the alteration of the existing binding and/or targeting domain or the substitution of same for a domain that has the required functionality.

It will be apparent from the description that in simple terms a calycin can be functionally divided into a “binding domain” and a “targeting domain”. The “binding domain” functions to interact with ligands and the “targeting domain” functions to provide specificity in transporting the bound ligand to a defined site. In some instances the binding domain may also be part of the targeting mechanism. Further, the subunit interaction domain functions to allow calycin multimerisation.

According to a further embodiment or aspect of the invention there is provided a calycin wherein said binding domain is adapted to bind more than one ligand and ideally said ligands are of a disparate nature according to the corresponding binding sites which are ideally based on different binding domains found in different calycins.

Methods for creating the above described calycins are well known in the art and comprise recombinant DNA techniques in the creation of calycin mutant and fusion proteins. It will also be apparent to those skilled in the art that the affinity of a calycin for a ligand can be altered, for example, by genetically modifying the ligand binding domain, to create an adapted calycin that has a higher affinity for a ligand. Moreover, the ligand binding domain may be genetically modified in this way to alter the specificity of ligand binding. It is also apparent that specificity of the targeting domain can be altered to either alter the specificity of targeting, or alternatively, increase or decrease the affinity of the targeting domain for its binding site. Genetic modification of this type is well known in the art and include, for example, the introduction of point mutations to alter the properties of the ligand binding site and/or the targeting domain.

It is also apparent that the production of molecular complexes with more than one type of ligand binding domain can be produced. This can be achieved by the fusion of

genes for the calycins to one another, with appropriate linking regions to produce a multi component gene and gene product. Or, interaction sites can be introduced into individual monomers of the calycins such that on mixing the individual proteins, molecules assemble into multi-sub unit complexes with similar or different functionalities. Genetic modifications of this type are well known in the art and include the introduction of point mutations, additions, deletion etc. to alter the properties of the protein. Additionally or alternatively, calycin monomers can be crosslinked to form multimeric complexes by chemical methods, including bifunctional cross-linking agents.

In yet a further preferred embodiment of the invention there is provided a calycin according to the invention wherein said calycin is manufactured using recombinant genetic techniques.

According to a further aspect of the invention there is provided a hair or skin care composition comprising at least one calycin according to the invention.

In a preferred embodiment of the invention said composition comprises at least one calycin characterised in that it has the ability to bind fatty acids that coat hair cuticles and or the skin surface or protein moieties that comprise the cuticle and/or skin surface. More preferably still, said composition is further characterised in that it binds at least one ligand, the targeting of which to the hair has a beneficial effect.

In yet a further preferred embodiment said beneficial effect is a therapeutic or cosmetic effect.

It will be apparent to one skilled in the art that said cosmetic effect involves the targeting of said calycin to hair or skin to provide a conditioning effect and moreover, as an alternative to or in combination with this, the targeting for example, of perfume or dye to hair or skin to provide at least one desired effect.

The term 'conditioning' is used herein with respect to hair to include, without limitation, detangling, softening and/or glossing effects. The term is used with respect to skin, to include, also without limitation, moisturising and softening effects.

It will be apparent to one skilled in the art that said therapeutic effect involves the targeting of said calycin to hair and/or skin to prevent and/or cure a medical condition.

It will be apparent to one skilled in the art that an example of a calycin having a therapeutic effect is a calycin that binds and transports an insecticide to hair and/or skin to prevent and/or cure infestation.

In a preferred embodiment of the invention said calycin composition is a veterinary composition for use in the treatment of parasitic infection of animals and/or birds.

In a further aspect of the invention there is provided a method for the treatment of humans and/or animals and/or birds which involves the administration of the therapeutic composition of the invention to an individual and/or animal and/or bird to prevent and/or cure a condition affecting hair, fur, hide, feathers, scalp and/or skin.

It will be apparent that the calycin provides a biodegradable binding and/or targeting means to deliver agents to hair.

An embodiment of the invention will now be described, by example only, with reference to the following figures, methods and materials wherein;

Figure 1 represents a diagrammatic representation of conserved characteristics found in many calycins.

10

Figure 2 represents a histogram showing the binding specificity of iodinated  $\beta$ -lactoglobulin ( $\beta$ -LG) for the hair cuticle.

Figure 3 represents an autoradiograph showing 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)-induced  $\beta$ -Lactoglobulin/recombinant Major Urinary Protein ( $\beta$ -LG/rMUP) heterodimer formation wherein;

Lane 1 represents  $\beta$ -LG and rMUP incubated with 0.5 mM EDC

Lane 2 represents rMUP incubated with 0.5 mM EDC

Lane 3 represents  $\beta$ -LG incubated with 0.5 mM EDC

M indicates a gel lane containing molecular mass markers.

Figure 4 represents an autoradiograph showing further evidence of EDC-induced  $\beta$ -LG/rMUP heterodimer formation wherein;

Lane 1 represents [ $^{35}$ S]Met-labelled rMUP +  $\beta$ -LG with 0.5 mM EDC

Lane 2 represents [ $^{35}$ S]Met-labelled rMUP with 0.5 mM EDC

Lane 3 represents [ $^{35}$ S]Met-labelled rMUP, untreated

M indicates a gel lane containing molecular mass markers.

Figure 5 represents an autoradiograph showing EDC-induced oligomerisation of  $\alpha$ -Crustacyanin wherein M indicates a gel lane containing molecular mass markers; and

Figure 6 represents an analysis of wash samples following [ $^{35}$ S]-rMUP (recombinant Major Urinary Protein) binding to hair. Labels on the abscissa refer to the following samples:

Not bound:	Cpm in the initial binding mixture after removal from hair
Wash 1:	Cpm in first wash with 100 $\mu$ L PBS
Wash 2:	Cpm in second wash with 100 $\mu$ L PBS
1% SDS Wash:	Cpm recovered in 100 $\mu$ L 1% SDS following incubation with the hair samples for 16 hr at 37°C
Still Hair Bound:	Cpm remaining bound to hair fibres after all washing steps

Values shown are the means of quadruplicate determinations and error bars represent S.E.M.

## **Materials and Methods**

### **Radioiodination of $\beta$ -lactoglobulin**

$\beta$ -lactoglobulin was radiolabelled with Iodobeads (Pierce) and  $\text{Na}^{125}\text{I}$ , according to the manufacturers instructions.  $18\mu\text{g}$  ( $1\text{nmol}$ )  $\beta$ -lactoglobulin in  $18\mu\text{l}$  water was incubated for 5 minutes at room temperature with  $20\mu\text{l}$  ( $1\text{mCi}$ ) carrier free  $\text{Na}^{125}\text{I}$  in the presence of two washed Iodobeads in  $200\mu\text{l}$  of  $100\text{mM}$  sodium phosphate, pH 6.5. The reaction was terminated by removal of the Iodobeads with forceps. Ovalbumin, ( $0.2\%$  w/v) in  $100\text{mM}$  phosphate pH7.4,  $0.14\text{M}$  NaCl (PBS) was added to the reaction mixture to bring the volume up to  $0.5\text{ml}$ . Gel filtration was performed using Sephadex G-50 ( $30\times 0.9$ ) cm, equilibrated and developed with  $0.2\%$  (w/v) ovalbumin in PBS, immediately after the labelling reaction to remove excess  $\text{Na}^{125}\text{I}$ .

### **Assay for $^{125}\text{I}$ $\beta$ -Lactoglobulin Binding to Fatty Acids on the Surface of Hair**

The binding of [ $^{125}\text{I}$ ] $\beta$ -lactoglobulin to cuticle cells was assayed in triplicate using an oil centrifuge method adapted from Sivaprasadorao (1987).  $5\text{mg}$  of isolated hair were mixed with [ $^{125}\text{I}$ ]- $\beta$ -lactoglobulin in  $100\mu\text{l}$  of assay buffer ( $20\text{mM}$  sodium phosphate, pH 7.4,  $150\text{mM}$  NaCl). After incubation for 15 mins at  $22^{\circ}\text{C}$ , the mixture was centrifuged for 2mins at  $12500\times g$  followed by removal of the supernatant. A series of washes with centrifugation as above, were carried out to remove non-specifically bound [ $^{125}\text{I}$ ]- $\beta$ -lactoglobulin:

**1<sup>st</sup> wash:**  $2\times 0.5\text{ml}$   $50\text{mM}$  Tris-Cl pH7.4,  $0.5\text{M}$  NaCl (high salt wash)

**2<sup>nd</sup> wash:**  $2\times 0.5\text{ml}$   $50\text{mM}$  Tris-Cl pH7.4 (low salt wash)

The cuticle sample was then overlaid with  $300\mu\text{l}$  of dibutyl phthalate. Bound [ $^{125}\text{I}$ ]  $\beta$ -

lactoglobulin was separated from unbound material by centrifugation for 2 mins at 12500xg. The bottoms of tubes containing the cuticle pellet were cut off after freezing in a dry-ice bath, drained on a paper towel and then measured for radioactivity in a LKB Rack gamma counter. This gave total binding. Parallel incubations in the presence of a 10000-fold excess of unlabelled  $\beta$ -lactoglobulin were carried out to determine non-specific binding. Specific binding was calculated by subtracting the non-specific binding from total binding.

### **Methods for generation of calycin multimers**

There are a number of possible approaches to generating multimers of calycin molecules. Multimers generated can be homomeric or heteromeric and may comprise two calycin molecules (dimers) or higher order complexes (trimer and above). Preferred routes to generate heterodimers by chemical means are outlined below.

### **Heterodimers cross-linked via the N-termini**

#### **Disuccinimidyl-suberimidate-dihydrochloride (DSS).**

DSS is an homobifunctional reagent (*N*-hydroxysuccinimide ester) which reacts with primary amine groups in proteins linking them via their N-terminal amine or surface-exposed lysine amino groups. DSS was prepared as a fresh stock solution of 20mg/ml in ice-cold 25mM  $\text{Na}_2\text{HPO}_4$ /1mM- $\text{MgCl}_2$  (pH 8.0) and added to a 1mg/ml calycin preparation in the same buffer to give a working concentration of 0.5, 2.0 and 10mg/ml. The reactions were allowed to proceed at room temperature (21°C) for 1 hour and quenched by the addition of 50 $\mu$ l of 1.0M-ammonium acetate per ml of reaction mixture.

#### **Dimethyl-adipimidate-dihydrochloride-(DMA)**

Conditions were identical to DSS treatment.

**1,5-difluoro-2,4-dinitrobenzene (DFDNB)**

DFDNB was prepared as a 1.0M stock solution and added to the calycin suspension in 25mM Na<sub>2</sub>HPO<sub>4</sub> /1mM MgCl<sub>2</sub> (pH 8.0) to give a final concentration of 5.0mM.

The reaction was terminated after 30 minutes at room temperature by dialysis against a large volume of 0.1M ammonium acetate (pH7.0).

***N*-hydroxysuccinimidyl 2,3-dibromopropionate (SDBP)**

SDBP is a heterobifunctional reagent (dibromopropionate and *N*-hydroxysuccinimide ester) used in sequential reactions to form the cross-link between the calycins. SDBP was prepared as a stock solution according to the manufacturer's instructions. The first calycin at a concentration of 1mg/ml was reacted with the *N*-hydroxysuccinimide moiety of SDBP added with constant stirring in phosphate buffer at pH 7 at 4°C for 1hr at an optimal concentration of SDBP. Excess cross-linker was removed by gel filtration and the derivatised calycin (now containing alkyl dibromide groups on its surface) was reacted with the second calycin (total protein concentration of 1mg/ml) by elevation of the temperature to 21°C. This reaction was also carried out with constant stirring under the same buffer and pH conditions as for the first reaction.

**Heterodimers cross-linked via amino and carboxyl groups****1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)**

1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) is used to catalyse the formation of an amide bond between the N-terminal amino group (or side chain amino group of lysine) of one calycin and the C-terminal carboxyl group of a second calycin to form the desired heterodimer. EDC was prepared as a stock solution according to the manufacturer's instructions. Protein was suspended to a concentration of 1.0mg/ml in 2-[*N*-morpholino]-ethanesulphonic acid (MES) buffered saline at pH 4.5-5.0 and EDC added with stirring for 16 hr at 25°C at an optimal concentration of EDC.

**Heterodimers cross-linked via one amino group and one free cysteine residue.****Succinimidyl 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate (SMCC)**

Succinimidyl 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate (SMCC) is a heterobifunctional cross-linking agent (*N*-hydroxysuccinimide ester and maleimide) that is used to sequentially react with the N-terminal amino group (or side chain amino group of lysine) of one calycin and a free sulphhydryl group on the other. SMCC was prepared as a stock solution according to the manufacturer's instructions. The reaction with the *N*-hydroxysuccinimide moiety was carried out in phosphate buffer at pH 7.0 with constant stirring at 4°C for 60 min at an optimal concentration of SMCC. Excess cross-linker was removed by gel filtration and the derivatised calycin (now with attached maleimide moiety) reacted with a surface-exposed cysteine side chain on the second calycin. The maleimide reaction was performed in phosphate buffer at pH 6.5-7.5 with constant stirring at 4°C for 20 hr with a final protein concentration of 0.1-1.0 mg/ml. This reaction is preferred where the first calycin lacks a free cysteine and the second calycin contains one.

Mixtures of undesirable calycin homodimers and the desired calycin heterodimers are isolated by a combination of two affinity chromatography steps that recognise the binding properties of both calycins. For most of the cross-linking agents outlined above analogues are available with different spacer arm lengths that could be used as necessary.

In general, for most of the cross-linking reactions outlined above the cross-linking reagents are used in 2-50 fold molar excess over protein; the actual concentration of cross-linker employed would also depend upon the protein concentration used. After completion of a cross-linking reaction excess or hydrolysed cross-linker is quenched and removed by gel filtration, dialysis or centrifugal concentration according to well established practices in the art. The exact conditions of any particular cross-linking



reaction, in terms of the reaction pH, temperature, time and concentration of protein and cross-linker, would need to be optimised for that particular reaction through experimentation.

#### **Formation of Heterodimers between $\beta$ -Lactoglobulin and Major Urinary Protein**

The cross-linking reagent, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), was used to effect the cross-linking of two calycons,  $\beta$ -Lactoglobulin ( $\beta$ -LG) and recombinant Major Urinary Protein (rMUP).

$\beta$ -LG(0.5 mg/mL) and rMUP(0.5 mg/mL) were incubated together and separately with 0.5 mM EDC at pH 4.5 for 22 hr at room temperature and then samples prepared for SDS-gel electrophoresis. The Coomassie blue stained gel is shown in Figure 3. The arrow indicates a heterodimer of  $\beta$ -LG and rMUP. M indicates a gel lane containing molecular mass markers.

Figure 3, lane 3 shows that  $\beta$ -LG alone, upon incubation with EDC, is not readily cross-linked to form homodimers. rMUP however (see Figure 3, lane 2) does form homodimers (approx. molecular mass, 45 kDa) in the presence of this cross-linker. When both these calycons are incubated together in the presence of EDC (Figure 3, lane 1) rMUP homodimer is still formed, but an additional band of approx. molecular mass 42 kDa is seen and indicates the formation of rMUP/ $\beta$ -LG heterodimers.

Further evidence for the formation of rMUP/ $\beta$ -LG heterodimers is demonstrated in Figure 4, which shows EDC-induced cross-linking of radiolabelled rMUP to  $\beta$ -LG. [ $^{35}$ S]Met-labelled rMUP (~100,000 cpm) was incubated with 0.5 mM EDC at pH 4.5 for 22 hr at room temperature either alone or with 0.5 mg/mL  $\beta$ -LG. Samples were then analysed by SDS-gel electrophoresis and the dried gel exposed to autoradiography film which is shown in Figure 4.

The autoradiograph depicted in Figure 4 shows that rMUP alone (present at vastly

lower concentration than in the experiment shown in Figure 3) did not produce sufficient homodimer for detection. However, the incubation of the same amount of radiolabelled rMUP with an excess of  $\beta$ -LG resulted in heterodimer formation as indicated by the labelled band at 42 kDa.

#### **Formation of Homodimers, Heterodimers and Oligomers from $\alpha$ -Crustacyanin**

Native  $\alpha$ -Crustacyanin (~0.3 mg/mL) isolated from lobster tissues was incubated with various concentrations of EDC ranging from 20  $\mu$ M to 10 mM at pH 4.5 for 20 hr at room temperature. Samples were analysed by SDS-gel electrophoresis. The resulting Coomassie blue stained gel is shown in Figure 5.

Figure 5 shows that this calycin is very efficiently cross-linked to form very large oligomeric species which are likely to reflect the oligomeric nature of the native protein itself, i.e. proposed to be an octomer of non-covalently associated heterodimers comprised of monomers, C1 and A2.

Figure 5 shows the two monomers clearly resolved in the untreated sample and as the concentration of the EDC cross-linker is increased, the A2 monomer is preferentially cross-linked followed by the A2 subunit into oligomers of increasing size. With 10 mM EDC virtually all the monomer is cross-linked and small amounts of dimers are visible.

#### **Binding of Major Urinary Protein (MUP) to Hair Fibres**

Six histidine tagged [ $^{35}$ S]Met-labelled rMUP was produced in *E. coli*, purified on Ni-NTA resin and used to assess binding of this protein to human hair fibres. Aliquots of [ $^{35}$ S]Met-labelled rMUP (c.5,000 cpm) were incubated at room temperature for one hour with c.20 mg portions of human hair fibre in a final volume of 100  $\mu$ L 25 mM sodium phosphate, pH 7.2, 150 mM NaCl (PBS) in the presence (non-specific binding) or absence (total binding) of 20  $\mu$ M unlabelled rMUP. Total binding, in the presence of [ $^{35}$ S]rMUP only, and non-specific binding, in the presence of a vast molar excess

17

(>1,000-fold) of unlabelled rMUP were determined following various washing steps. The amounts of [ $^{35}\text{S}$ ]rMUP recovered in each washing solution were determined, as well as the amount of [ $^{35}\text{S}$ ]rMUP remaining bound to the hair fibres after washing with 1% SDS.

## **Results**

### **Assay for $^{125}\text{I}$ $\beta$ -Lactoglobulin Binding to Fatty Acids on the Surface of Hair**

A 1000-fold excess of unlabelled  $\beta$ -lactoglobulin competitively displaced approximately 20% of [ $^{125}\text{I}$ ] $\beta$ -lactoglobulin bound to the cuticle surface, see Table 1.

Table 1

Total binding (A) $^{125}\text{I}$ $\beta$ -lactoglobulin	Non-specific binding (B) $^{125}\text{I}$ $\beta$ - lactoglobulin	Specific-binding (A-B)
10838cpm ( 0.005 $\mu\text{Ci}$ )*	8574cpm ( 0.004 $\mu\text{Ci}$ )	2264cpm (0.001 $\mu\text{Ci}$ )

\* Errors in these experiments are less than +/- 10%

### **Binding of Major Urinary Protein (MUP) to Hair Fibres**

Figure 6 shows that with the early washing steps, significantly more [ $^{35}\text{S}$ ]rMUP is removed in the samples containing an excess of unlabelled MUP (i.e. the non-specific binding samples) than in the total binding samples. Washing with 1% SDS would be expected to remove all the hair bound [ $^{35}\text{S}$ ]rMUP and hence these samples represent [ $^{35}\text{S}$ ]rMUP bound to hair.

Radioactive counts in these samples indicate that, of the total [ $^{35}\text{S}$ ]rMUP binding, ~31% is specifically bound. Interestingly, some [ $^{35}\text{S}$ ]rMUP remained hair bound even after washing with SDS and more remained bound in the total binding samples than

18  
in the non-specific samples. This indicates particularly strong adsorption to the tissue.

### **Conclusions**

It is concluded from these results that  $\beta$ -lactoglobulin binds specifically to the hair surface. The hydrophobic nature of the ligand binding site on the protein suggests that non-polar moieties on the cuticular surface may act as ligands for  $\beta$ -lactoglobulin. A prime example of this may be the cuticle. The rather non-selective nature of the  $\beta$ -lactoglobulin binding site provides potential for protein engineering to produce much more effective interaction with the cuticle and hence hair fibre surface.

The data presented here show that calycin monomers can be readily cross-linked into homodimers, heterodimers and higher oligomers. Such reactions will allow the formation of calycin species that possess two or more distinct and specific binding pockets. The degree to which homodimers, heterodimers and oligomers are formed is dependent on the specific calycin(s), for example, MUP is readily cross-linked by EDC to form homodimers whereas  $\beta$ -LG is not. Where the calycin monomers are initially in close non-covalent association with each other, as in the case of  $\alpha$ -Crustacyanin, very efficient cross-linking can be achieved.

In addition to  $\beta$ -LG binding of human hair cuticle preparations, the results also demonstrate the specific binding of another calycin, rMUP, to human hair fibres.

Small amounts of rMUP binding to hair fibres are even resistant to an extensive washing with SDS - a common component of most shampoo preparations.

**CLAIMS**

1. A calycin comprising a binding domain for binding at least one selected agent and a targeting domain that binds to at least a part of a hair fibre and/or skin surface for targeting said calycin to said hair fibre and/or skin surface.
2. A calycin according to claim 1, further comprising a subunit interaction domain wherein said subunit interaction domain is a naturally occurring part of the calycin or is inserted using conventional synthetic or recombinant techniques.
3. A calycin multimer formed by the multimeric assembly of calycins according to claim 2
4. A calycin according to claim 1 or claim 2 or a calycin multimer according to claim 3 wherein said binding domain and/or said targeting domain is endogenous to the calycin or calycin multimer or is a corresponding binding and/or targeting domain which is adapted accordingly by alteration of the existing binding and/or targeting domain or the substitution of the existing binding and/or targeting domain for a domain that has the required functionality.
5. A calycin or a calycin multimer according to any of claims 1 to 4 wherein said binding domain is adapted to bind more than one ligand.
6. A calycin or a calycin multimer according to claim 5 wherein the ligands are of a disparate nature according to the corresponding binding sites which are based on different binding domains found in different calycins.
7. A calycin or a calycin multimer according to any of the preceding claims wherein the or at least one calycin is  $\beta$ -lactoglobulin.

8. A calycin or a calycin multimer according to any of the preceding claims wherein the or at least one calycin is Major Urinary Protein and/or recombinant Major Urinary Protein.
9. A method for creating a calycin or a calycin multimer according to any of the preceding claims wherein said method comprises the use of recombinant DNA techniques in the creation of calycin mutant and fusion proteins.
10. A method according to claim 9 wherein the ligand binding domain and/or the targeting domain is genetically modified to alter the specificity of ligand binding and/or the affinity of the targeting domain for its binding site.
11. A method according to claim 9 or claim 10 further comprising the production of molecular complexes with more than one type of ligand binding domain wherein calycin genes are fused to one another and/or appropriate linking regions are used to produce a multi component gene and gene product and/or interaction sites are introduced into individual monomers of the calycins such that on mixing the individual proteins, molecules assemble into multi-sub unit complexes with similar or different functionalities.
12. A method according to claim 11 wherein said method additionally or alternatively comprises chemical methods in the crosslinking of calycin monomers to form multimeric complexes.
13. A method according to claim 12 wherein said chemical methods include bifunctional cross-linking agents.
14. A method according to claim 13 wherein the cross-linking agent is 1-ethyl-3-[3-

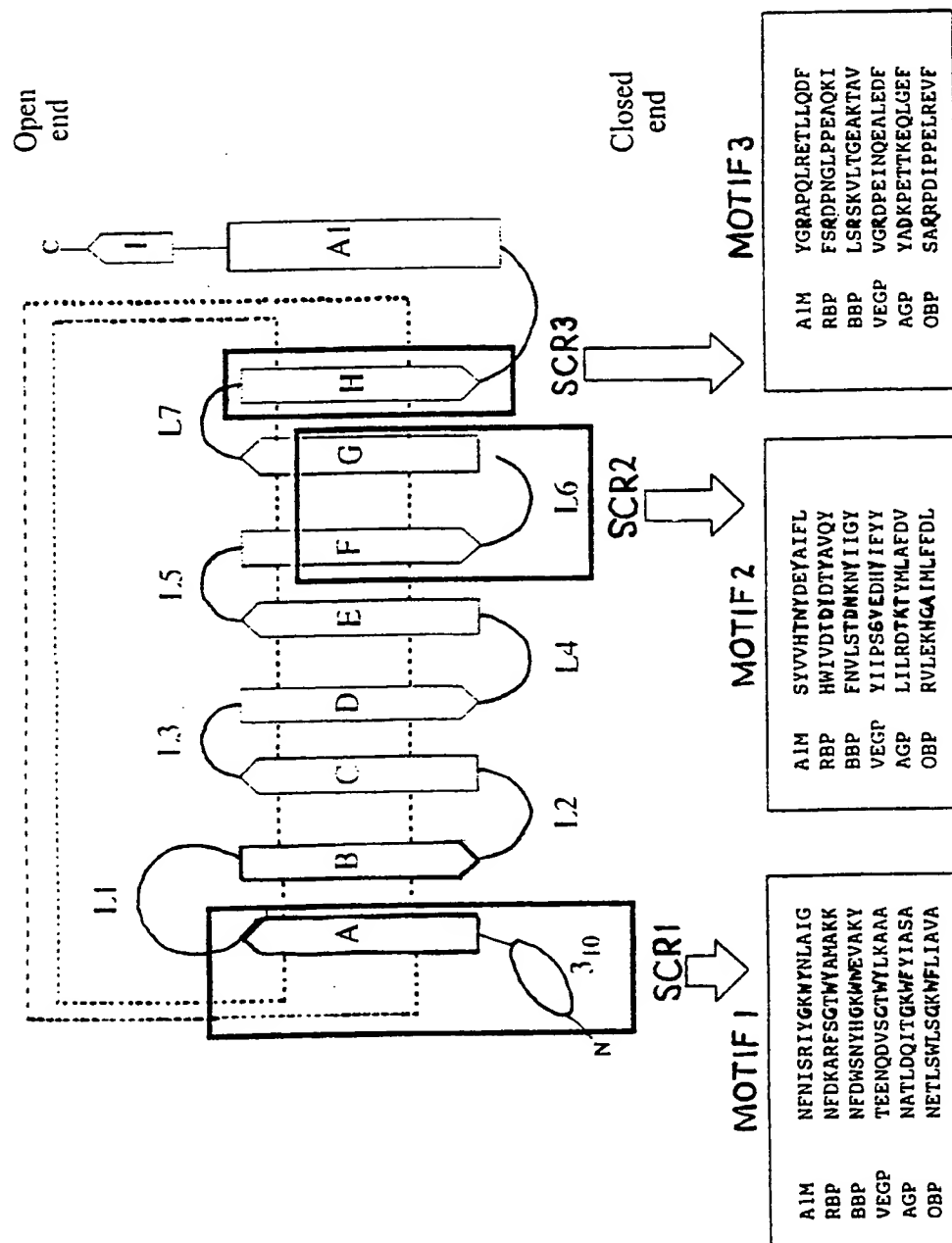
dimethylaminopropyl]carbodiimide hydrochloride (EDC).

15. A method according to any of claims 9 to 13 to effect the cross-linking of  $\beta$ -Lactoglobulin and recombinant Major Urinary Protein.
16. A calycin or a calycin multimer according any of claims 1 to 8 wherein said calycin or calycin monomer is manufactured using recombinant genetic techniques and/or chemical methods according to any of claims 6 to 15.
17. A hair or skin care composition comprising at least one calycin according to any of claims 1 to 8 and/or claim 16.
18. A composition according to claim 17 wherein said composition comprises at least one calycin characterised in that it has the ability to bind fatty acids that coat hair cuticles and/or skin or protein moieties that comprise the cuticle and/or skin.
19. A composition according to claim 17 or 18 wherein said composition provides a cosmetic or therapeutic effect.
20. A composition according to claim 19 wherein the cosmetic effect involves the targeting of said calycin to hair to provide a conditioning effect and/or the targeting of perfume or hair dye to hair to provide at least one desired effect.
21. A composition according to claim 19 wherein the therapeutic effect involves the targeting of said calycin to hair and/or skin to prevent and/or cure a medical condition.
22. A composition according to claim 21 wherein the calycin binds and transports an insecticide to hair and/or skin to prevent and/or cure infestation.

23. A composition according to claims 21 or 22 which is a veterinary composition for use in the treatment of parasitic infection of animals and/or birds.
24. A method for the treatment of humans and/or animals and/or birds which involves the administration of the therapeutic composition of the invention to an individual and/or animal and/or bird to prevent and/or cure a condition affecting hair, fur, hide, feathers, scalp and skin.



FIGURE 1;



# Histogram showing binding of [ $^{125}$ I] $\beta$ -lactoglobulin to the hair cuticle

FIGURE 2;

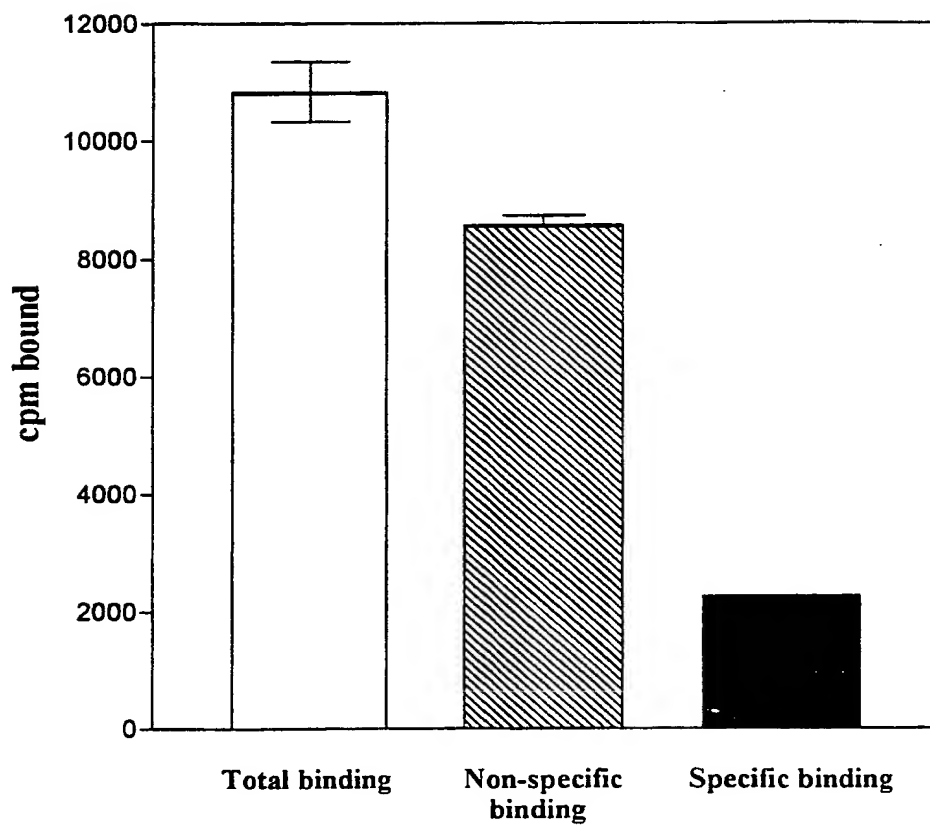


Figure 3;

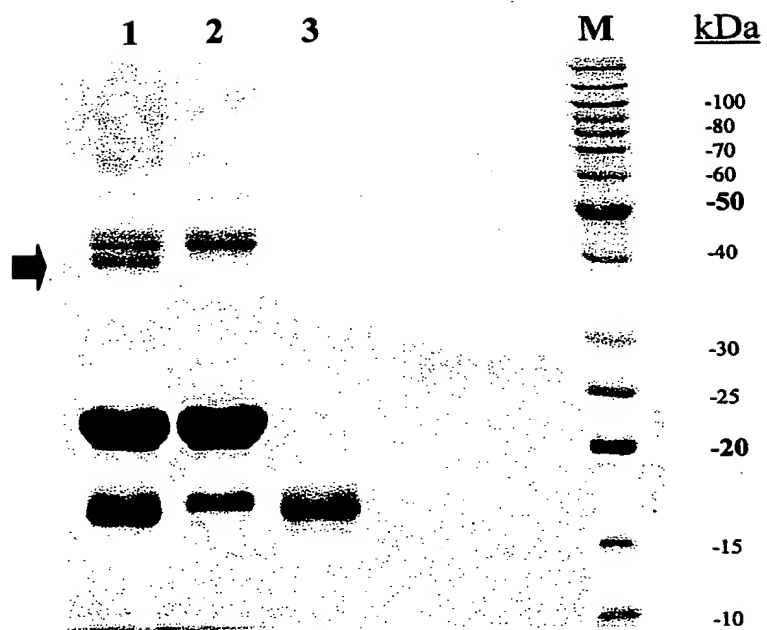


FIGURE 4;

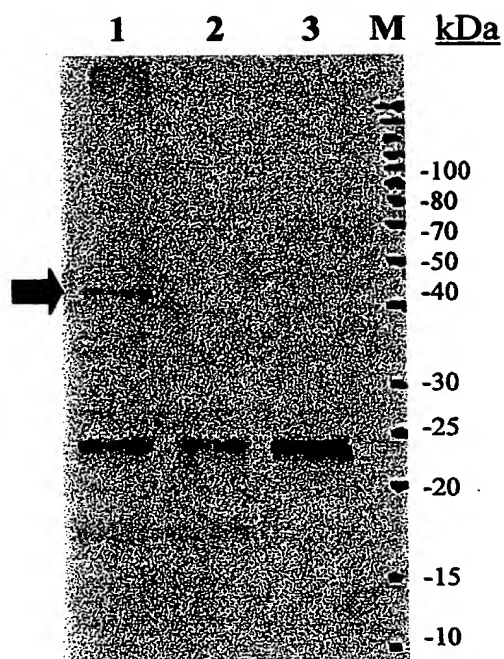


FIGURE 5;

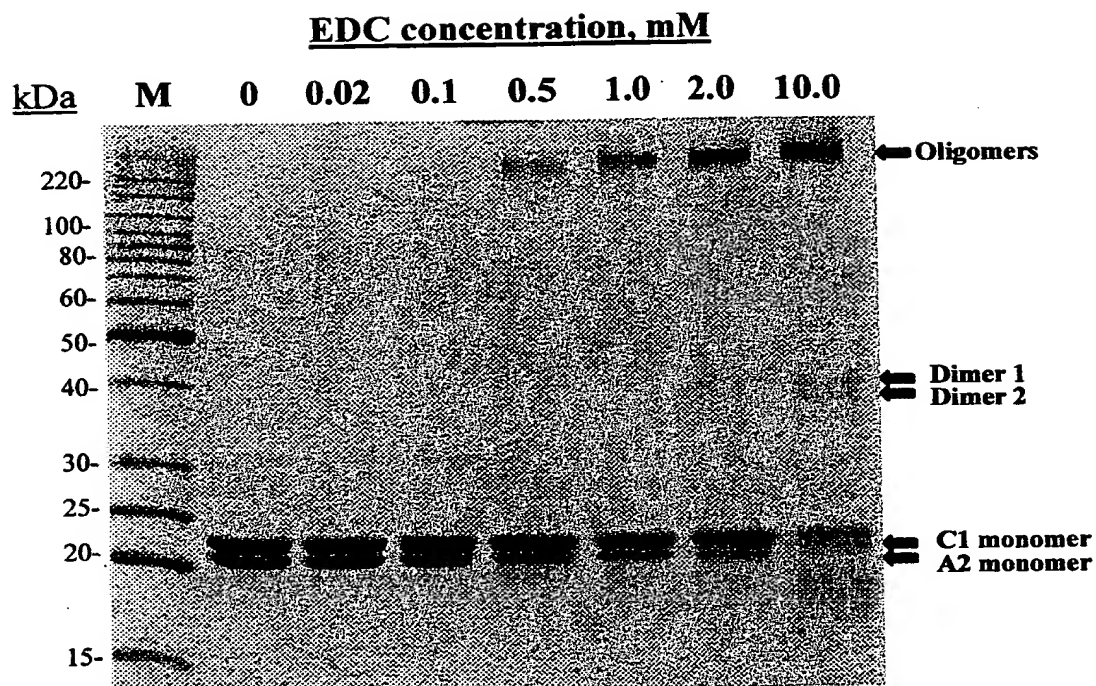
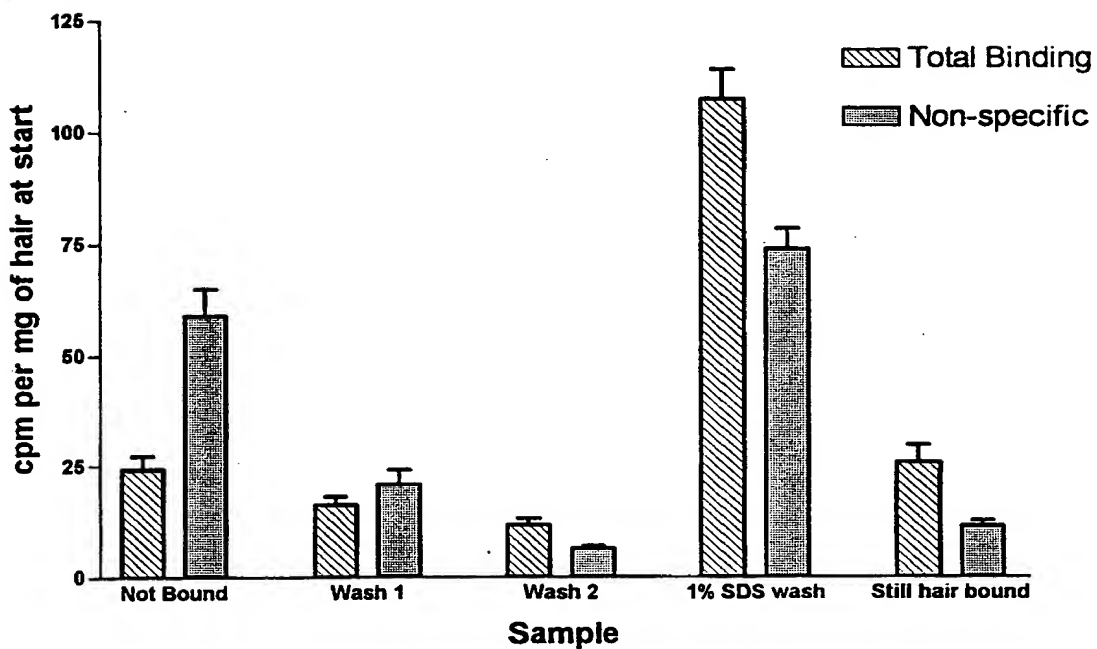


FIGURE 6;  
Analysis of wash samples following [ $^{35}$ S]-rMUP  
binding to hair\*



Washes 1 & 2: 25 mM Phosphate pH 7.4, NaCl 150 mM

\*NB. Data corrected for weight of hair used in each sample at start

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 00/00517

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K7/06 A61K7/48 A61K7/46 A61K7/13 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	CHATEL ET AL.: "Expression of a Lipocain in Prokaryote and Eukaryote cells..." PROTEIN EXPRESSION PURIFICATION, vol. 16, no. 1, June 1999 (1999-06), pages 70-75, XP000913408 abstract	1,2,4,7, 16
Y,P	---	4-6,9-15
Y,P	WO 99 16873 A (SKERRA ET AL.) 8 April 1999 (1999-04-08) page 8, line 22 - line 37 page 22, line 17 -page 23, line 23 claims 1-10 --- -/-	4-6,9-11

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

15 June 2000

Date of mailing of the international search report

06/07/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Alvarez Alvarez, C

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 00/00517

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZAGALSKY ET AL.: "The quaternary structure of the lobster carapace..." COMP. BIOCHEM. PHYSIOL., B: COMP. BIOCHEM., vol. 97B, no. 4, 1990, pages 837-848, XP000913411 abstract page 838 "Cross-linking"	1-4
Y	---	11-15
Y	LEE ET AL.: "Crosslinking of tissue-derived biomaterials..." JOURNAL MATERIALS SCIENCE, vol. 7, no. 9, 1996, pages 531-541, XP000907368 abstract	14
X	VERHEUL ET AL.: "Association behavior of native beta-lactoglobulin" BIOPOLYMERS, vol. 49, no. 1, 1999, pages 11-20, XP002140160 "Conclusions"	1-4,7
X	SHAHAN ET AL.: "Expression of six mouse major urinary protein genes ..." MOL. CELL. BIOL., vol. 7, no. 5, 1987, pages 1947-54, XP000913409 abstract	8
X	US 5 679 329 A (DUPUIS ET AL.) 21 October 1997 (1997-10-21) column 2, line 3 - line 52; claim 1	17-19
X	CH 686 998 A (PARFUMS GIVENCHY) 30 August 1996 (1996-08-30) page 2, line 47 - page 3, line 55	17-19
X	EP 0 850 642 A (L'OREAL) 1 July 1998 (1998-07-01) page 5, line 31 - line 44; claim 1	17-19



# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/GB 00/00517

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9916873 A	08-04-1999	DE 19742706 A AU 1143799 A	15-04-1999 23-04-1999
US 5679329 A	21-10-1997	FR 2688135 A AT 137110 T CA 2131688 A DE 69302375 D DE 69302375 T EP 0632715 A ES 2086936 T WO 9317656 A JP 2860162 B JP 7504424 T	10-09-1993 15-05-1996 16-09-1993 30-05-1996 05-09-1996 11-01-1995 01-07-1996 16-09-1993 24-02-1999 18-05-1995
CH 686998 A	30-08-1996	NONE	
EP 850642 A	01-07-1998	FR 2757767 A JP 10212226 A US 5908618 A	03-07-1998 11-08-1998 01-06-1999

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**